Tarlton, J. F., Wilkins, L. J., Toscano, M. J., Avery, N. C., & Knott, L. (2013). Reduced bone breakage and increased bone strength in free range laying hens fed omega-3 polyunsaturated fatty acid supplemented diets. *Bone*, *52*(2), 578-586. https://doi.org/10.1016/j.bone.2012.11.003

Reduced bone breakage and increased bone strength in free range laying hens fed omega-3 polyunsaturated fatty acid supplemented diets

John F. Tarlton[a *], Lindsay J. Wilkins[b], Michael J. Toscano[a], Nick C. Avery[a] and Lynda Knott[a].

[a] *Matrix Biology Research Group, University of Bristol, Langford, Bristol BS40 5DU, UK.*
[b] *Animal Behaviour and Welfare Group, University of Bristol, Langford, Bristol BS40 5DU, UK.*

**Corresponding Author at: Matrix Biology Research Group, University of Bristol, Langford, Bristol BS40 5DU, UK.*
E-mail address: john.tarlton@bristol.ac.uk (John Tarlton)

Abstract

Introduction: The omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) are the immediate precursors to a number of important mediators of immunity, inflammation and bone function, with products of omega-6 generally thought to promote inflammation and favour bone resorption. Western diets generally provide a 10 to 20-fold deficit in omega-3 PUFAs compared with omega-6, and this is thought to have contributed to the marked rise in incidence of disorders of modern human societies, such as heart disease, colitis and perhaps osteoporosis. Many of our food production animals, fed on grains rich in omega-6, are also exposed to a dietary deficit in omega-3, with perhaps similar health consequences. Bone fragility due to osteoporotic changes in laying hens is a major economic and welfare problem, with our recent estimates of breakage rates indicating up to 95% of free range hens suffer breaks during lay.
Methods: Free range hens housed in full scale commercial systems were provided diets supplemented with omega-3 alpha linolenic acid, and the skeletal benefits were investigated by comparison to standard diets rich in omega-6.
Results: There was a significant 40-60% reduction in keel bone breakage rate, and a corresponding reduction in breakage severity in the omega-3 supplemented hens. There was significantly greater bone density and bone mineral content, alongside increases in total bone and trabecular volumes. The mechanical properties of the omega-3 supplemented hens were improved, with strength, energy to break and stiffness demonstrating significant increases. Alkaline phosphatase (an osteoblast marker) and tartrate-resistant acid phosphatase (an osteoclast marker) both showed significant increases with the omega-3 diets, indicating enhanced bone turnover. This was corroborated by the significantly lower levels of the mature collagen crosslinks, hydroxylysyl pyridinoline, lysyl pyridinoline and Histidinohydroxy-lysinonorleucine, with a corresponding significant shift in the mature:immature crosslink ratio.
Conclusions: The improved skeletal health in laying hens corresponds to as many as 68 million fewer hens suffering keel fractures in the EU each year. The biomechanical and biochemical evidence suggests that increased bone turnover has enhanced the bone mechanical properties, and that this may suggest potential benefits for human osteoporosis.

1. Introduction

There are over 350 million laying hens in the EU, producing around 100 billion eggs. Approximately one third of hens are housed in barn or free range systems, with another third in furnished cages. These figures are likely to increase due to the 2012 EU directive banning battery cage systems. Our recent estimates of keel bone (sternum) breakage rates in free range systems have shown that up to 95% of laying hens may suffer bone breakage, probably from a combination

of bone weakness and hazardous housing conditions[1]. A substantial level of damage (62%) has also been identified in furnished cage systems[2], with an overall EU annual incidence of keel fractures in excess of 100 million. Skeletal health in laying hens is therefore a major welfare and economic problem, and seriously damages the public perception of egg production. This problem is made all the more urgent by the EU directive, with a potential for an "epidemic" of broken bones in free range and furnished cage systems post 2012.

Current interest in omega-3 (n3) polyunsaturated fatty acid (PUFA) supplementation in human diets results from evidence that a number of conditions prevalent in Western societies, such as heart disease[3], arthritis[4, 5], colitis[6] and osteoporosis[7], are due to 10 to 20-fold deficit in n3 PUFAs compared with n6, whereas natural human diets are thought to have approximately equal levels [8]. Similarly, it is thought that the natural foraging diet of hens would provide a relatively high level of n3 through consumption of green leaves, and therefore farmed laying hens, fed a grain rich diet, are also exposed to unnaturally low levels of n3. The source of the problem is similar for both humans and hens, that the modern food chain is based largely on cultivated grain, such as wheat, rice, corn, sunflower and soya, all of which have high levels of n6 and low or non-existent levels of n3. This results in diets with typically between 8 and 30 fold deficiency of n3 in relation to n6.

PUFAs are the immediate precursors to a number of mediators of inflammation and bone function such as prostaglandins (PG), leukotrienes, thromboxanes, resolvins and lipoxins, with n3s generating mediators which are less pro-inflammatory and more pro-inflammation resolving than those from n6 PUFAs, and with different influence on bone biology[9-11] (see supplementary Fig. S1). Therefore it is likely that promoting a natural balance of n3 and n6 in the food chain would improve human and food animal health, where there is a current imbalance. Bone fragility and breakage is a problem for both humans and farmed hens, and there is evidence from human and animal studies that a balanced n3/n6 dietary intake reduces signs of osteoporosis[7], probably by modulating prostaglandin activities. Therefore, there is potential to reduce rates of bone breakage in laying hens by dietary supplementation with n3 PUFA. By providing improved human nutrition through supplementing the diets of food animals, both will benefit from improved health.

This study aims to explore the hypothesis that n3 supplemented diets with a more natural balance of fatty acids will reduce the very high rates of keel bone breakage in commercial free range systems We will also explore mechanisms relating to bone strength, structure, mineral content and metabolism.

2. Materials and Methods

2.1. Hens, Diets and samples

We compared 6 commercial free range flocks fed, *ad libitum,* a standard diet high in 18:2n6 linoleic acid (LA), with 6 flocks fed a diet supplemented with 18:3n3 alpha linolenic acid (ALA, Optomega) from flaxseed (linseed) (Table 1). Diets were analysed for their fatty acid content using the Folch procedure[12]. The n3 diets were typically begun at 23 weeks of age and no later than 30 weeks. Assessments were made at three time-points, at 30, 50 and 70 weeks of age, with palpations typically 100/flock per time point, and samples collected 10 or 20/flock per time point. Although commercial considerations limited some data collections, this was accounted for in the study design. Though the housing design was not controlled for in this study, there was no systematic or significant difference in the quantifiable housing parameters between the diet treatments. At dissection, humeri, tibiae and keel bones were collected for analysis. The keel and the tibia were used for measurements of mechanical properties, and the humerus for biochemical analysis (Fig.S2). The tibia was used for analysis of material properties due to its regular geometry, but was not used for biochemistry due to problems of contamination from non-structural marrow in the medullary bone cavity. All procedures involving animals fell within nationally permissible

procedures, were in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and received all appropriate local ethical approvals.

Table 1. Compositions of diets

Raw Material	% composition	
	Standard diet	n3 Diet
MCP (P)		0.506
Wheat	39.2	50.4
Whole maize	25	
Wheatfeed P		5
Lucerne Conc		0.834
Field peas		5
Non GM Braz Soya	17.38	14.729
Soya oil	2.13	
Sunflower 32% P	5.23	3.26
Alimet P	0.142	0.107
Optomega 50 Lins+E		10
Greens		0.25
Choline Chl 60%		0.042
Multiphos TRI/DFP	0.794	
Limestone P	0.9	9.066
Limestone grit	8.1	
Vit E 50g/kg		0.028
Salgard SW Liquid	0.3	
Sodium bicarbonate P	0.142	0.145
Salt P	0.243	0.29
LC Lay+pigment P	0.333	0.333
Lysine HCL P	0.107	

2.2. Bone breakage assessments

Keel bone breakage was evaluated by palpation, as developed at the University of Bristol[13], on 100 birds per flock per time point, and confirmed by dissection on 10-20 birds/flock per time point. Previous results have shown palpation to have a predictive value of 0.95 of breaks identified at dissection[1]. On each visit, the house lights were turned down to minimise bird panics. At dissection, keel fracture severity was determined by comparison with an ordinal scale (Fig.1), where zero indicates no fracture, one a minor fracture, and increasing severity up to a score of four indicating severe or multiple fractures. Minor damage is mostly restricted to the tip of the keel. Increasing severity of damage is associated with greater calcification and callus formation with additional damage extending to the ventral edge of the keel. Multiple damage sites extend caudally and are associated with gross deformation[1].

Fig. 1

2.3. Bone mineral analysis

Quantification of bone mineral density (BMD) was performed on left humeri using dual energy X-ray absorptiometry (DEXA, Lunar PIXImus densitometer, Lunar Corp). Each bone was thawed to room temperature prior to testing, the length measured, the midsection marked, and a 0.08 cm^2 region of interest (ROI) was measured adjacent to the midsection of each bone (marked on the scan with the aid of a wire placed across the midsection) and the BMD recorded. The overall mineral content of bone was calculated as a percentage of humerus dry weight remaining after ashing in a muffle furnace at 700°C for 24 hours.

2.4. Histomorphometry

Tibial metaphyses, at 70 weeks, were fixed in neutral buffered formalin and decalcified for 6 weeks in 10% EDTA (pH 7·4). The samples were dehydrated, cleared in histoclear, and embedded in paraffin wax, and 6 µm sections cut from each block. Sections were stained with 1% toluidine blue and trabecular and medullary bone volumes and trabecular thickness were measured across whole sections using semi-automated image analysis (NIH-Image version 1·62).

2.5. Biomechanical properties

The left tibia underwent 3-point bending to failure using an Instron 6022 materials testing apparatus (Instron UK) as previously reported [14, 15]. In brief, bones were mounted across a supporting bridge with a gap of 36 mm, and perpendicular load applied to the centre point at a speed of 2.5mm/minute. The breaking stress, energy to break, plastic energy, elastic energy and Young's modulus were read or calculated from load-deformation outputs. For instance, for ultimate stress $\sigma=FLR/4I$, where F is the ultimate force, L is the distance between supports, R is the radius in the direction of load and I is the moment of area, where $I=\pi(A^3B - a^3b)/4$ (A and a are the outer and inner diameters in the direction of load and B and b those perpendicular to load)[15].

Keel bones were positioned on a custom support within a mechanical testing apparatus (Stevens CR analyser, Mechtric Engineering, UK), and loaded to failure at a constant speed of 50 mm/min using a 6.8mm blunt probe at the manubrial spine (A) and the lateral surface (B) (Fig. 1). The force required to reach structural failure was recorded. Due to its complex geometry, it was not possible to calculate material properties for the keel.

2.6. Alkaline phosphatise (ALP)

The humeri were pulverised, freeze-dried and extracted with 20µl/mg 0.1% Brij 35 in 20 mM triethanolamine at 4°C for 18h, and extract aliquots stored at -80°C until use and not exposed to multiple freeze-thaw cycles. Extracts were analysed for ALP activity, a marker of osteoblastic bone

formation, by conversion of *p*-nitrophenylphosphate (pNPP), to *p*-nitrophenol and monitored at 405nm using a Konelab analyser[16].

2.7. Tartrate resistant acid phosphatase (TRAP)

Bone extracts were analysed for levels of TRAP, a marker of osteoclastic bone resorption, colorimetrically by conversion of pNPP in the presence of disodium tartrate[17].

2.8. Pro-matrix metalloproteinase (MMP)-2

Humeri extracts (see 2.6) were assayed for MMP-2 activity, a marker of collagen turnover, by gelatin zymography as previously described[18]. In brief, aliquots of extracts were electrophoresed with MMP-2 standard (Calbiochem, Nottingham, UK) in 10% polyacrylamide gels containing 1mg/ml gelatin and incubated for 16 h at 37°C in MMP proteolysis buffer (50 mM Tris/HCl pH 7.8, 50 mM CaCl2, 0.5 M NaCl). Gels were stained with Coomassie blue, and proteolytic activity was quantified by optical scanning of the gels (Epson V700 Scanner) and subsequent computer analysis of the band intensities (Image J 1.41)[5, 18].

2.9. Collagen crosslinks

Mature hydroxylysyl-pyridinoline (HL-Pyr), lysyl-pyridinoline (L-Pyr) and histidinohydroxy-lysinonorleucine (HHL), plus intermediate hydroxylysino-5-ketonorleucine (HLKNL) and hydroxylysinonorleucine (HLNL) collagen cross-links were quantified (M/M collagen) using modified amino acid analysis as described previously[19]. Mature pyrrolic cross-links were analysed from heat denatured material after trypsin solubilisation, then reacted with 4-dimethylaminobenzaldehyde and compared with N-methylpyrrole, standard curve as previously described[20]. Collagen content (% dry weight) was determined by hydroxyproline analysis of a tissue hydrolysate using a continuous-flow autoanalyser (Burkard Scientific, Uxbridge, UK) as previously described[20, 21].

2.10. Statistical analysis

Treatment (diet) effects were modelled using MlwiN[22], a statistical package designed for hierarchical data analysis. Data was normalized by log transformation if needed following assessments of a normal distribution. The response was modelled with a structure of flock (j) containing bird (i). Flock age and treatment were initially included as fixed effects for all responses and then removed individually when comparison of the respective Z-ratio with a standard normal distribution was greater than 1.96 ($p>0.05$). Flock age was included as a polynomial term when appropriate following visual inspection of the data plots.

For comparisons between groups at each time point a one way ANOVA was used, with Bonferroni's Multiple Comparison Test where $p<0.05$, or a Kruskal-Wallis test with Dunn's Multiple Comparison Test where variances are unequal (Bartlett's test for equal variances). Graphical data is presented as mean ± 95% confidence interval.

3. Results

A comprehensive table of results is shown in supplementary material Table S1.

3.1. Polyunsaturated fatty acid (PUFA) composition of diets

The principle PUFA components of the diets were the C18 n6 linoleic acid (LA) and the C18 n3 alpha-linolenic acid (ALA) (Table 2). There was no detectable long chain C20 n6 arachidonic acid, the C20 n3 eicosapentenoic acid (EPA) or the C22 n3 docosahexanoic acid (DHA).

Table 2. Fatty acid analysis of diets

Fatty acid	% of total fatty acid	
	Standard diet	n3 diet
12:00	0.60	0.032
14:00	0.41	0.113
15:00	0.07	0.063
16:00	16.94	10.31
16:01	0.22	0.162
17:00	0.09	0.095
18:00	2.25	3.25
18:1n-7*trans*	0.16	0.626
18:1n-9	14.48	14.38
18:1n-7	1.09	0.748
18:2n6 (LA)	**50.60**	**27.19**
20:00	0.24	0.190
20:1n-9	0.28	0.210
18:3n3 (ALA)	**6.61**	**35.56**
18:04	0.00	0.00
20:2n6	0.13	0.52
22:00	0.27	0.20
20:3n6	0.00	0.00
20:4n6 (AA)	0.00	0.00
20:4n3	0.00	0.00
20:5n3 (EPA)	0.00	0.00
22:4n6	0.00	0.00
22:5n3	0.00	0.00
22:6n3 (DHA)	0.00	0.00

3.2. Keel bone breakage rates

The likelihood of fracture was analyzed using logistic regression with a log-link transformation where the response was the proportion of birds that were found to have breaks within each flock (j) and time point (i). The number of birds examined was used to weight the proportions. With this analysis, exponentiation of the associated model coefficients yields the relative odds of a fracture occurring with within n6 versus n3 flocks.

By dissection: Regression modelling of the dissection data shows that the n6 fed birds had an odds ratio of a fracture occurring 3.8 times greater over the entire laying period than the n3 birds ($p<0.001$). Bone breakage rates in standard n6 diet group were 3.3% (SEM ±2.1) at 30 weeks, 56.2% (±7.2) at 50 weeks and 62% (±5.6) at 70 weeks, with rates significantly lower in the n3 supplemented flocks at 50wks (21.7% ±3.1, $p<0.01$) and 70 weeks (38.3% ±5.9, $p<0.01$), with no significant difference in breakage rate at 30 weeks (5.2% ± 2.3, NS) (Fig. 2A). This represents a 60% reduction in breakage rates at 50 ($p=0.01$) weeks and a 40% reduction at end of lay ($p=0.001$). As well as reducing the number of breaks, n3 lowered the level of severity, with n3 having a significantly lower overall severity score at 50 and 70 weeks ($p<0.001$). Fracture occurence at 30 weeks was too low for statistical comparison of severity. When only fractured keels were considered, n3 had a treatment effect in reducing severity ($p<0.002$) (Fig. 2C). No flock effect was seen in either breakage rates or severity.

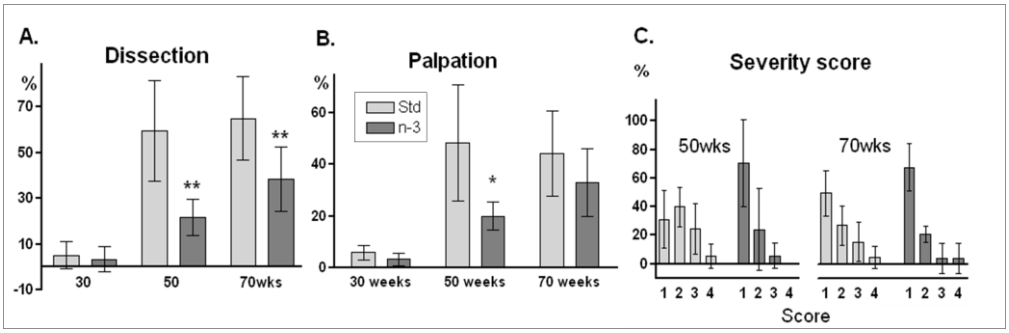

Fig. 2

By Palpation: Regression modelling using the palpation data shows that the n6 fed birds had an odds ratio of a fracture occurring 2.4 times greater over the entire laying period than the n3 birds (p=0.001). Consequently, the breakage rates determined by palpation were substantially lower in the n3 flocks compared with those fed a standard diet (Fig 2B). In standard diet free range systems breakage rates were 5.8% (±1.1) at 30 weeks, 48.4% (±8.1) at 50wks and 44.4% (±5.95) at 70wks. Rates were lower in the n3 supplemented flocks at 30 weeks (3.33% ±0.96, NS) 50wks (20.0% ±2.1, $p<0.05$) and 70 weeks (33.2% ±5.1, NS).

3.3. Mineral analysis

At 70 weeks there was greater BMD ($p<0.001$) in the humeri of the n3 supplemented flocks compared with the control n6 birds (Fig. 3A). This was corroborated by similar difference in mineral content ($p<0.0001$) at 70 weeks (Fig. 3B). There were no significant differences in either measure at 30 weeks.

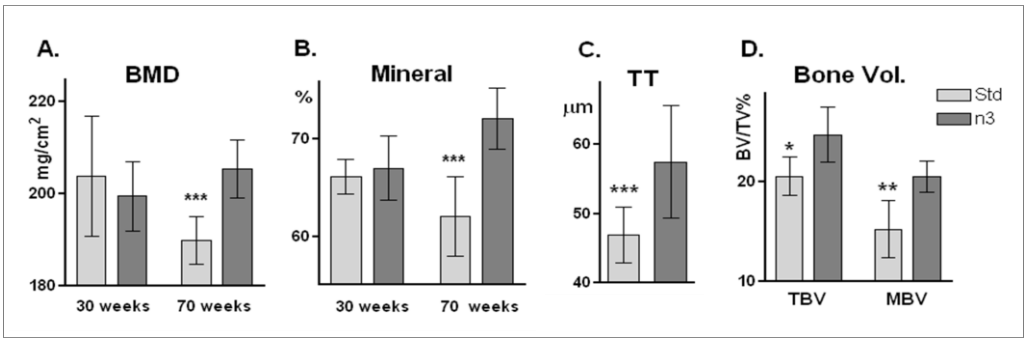

Fig. 3

3.4. Histomorphometry

Histomorhometry was performed at 70 weeks only. The trabecular thickness of the humeri were greater in the n3 group ($p<0.001$) (Fig. 3C). Both the trabecular ($p<0.02$) and medullary ($p<0.002$) bone volumes were greater in the n3 fed birds (ANOVA $p<0.0001$) (Fig. 3D).

3.5. Biomechanical properties

Breaking loads of the keel bone at the manubial spine (position “A” Fig. 1) was significantly greater in the n3 supplemented birds, with an overall treatment effect (p=0.0002) and greater strength at 70 weeks ($p<0.001$). At the lateral surface (position “B”) the keels were also stronger in the n3 group, with the overall diet showing an interaction with age and a significant effect at 70 weeks ($p<0.05$) and a time point comparison significant at 70 weeks ($p<0.01$).

The ultimate strength (stress), energy to break ("toughness") and stiffness (Young's modulus) of the tibia exposed to three point bending showed a significant treatment effect with the n3 birds having stronger (p=0.002), tougher (p=0.001) and stiffer (p<0.02) bones (Fig. 4). The significance of these differences at each time point is shown in Table 3.

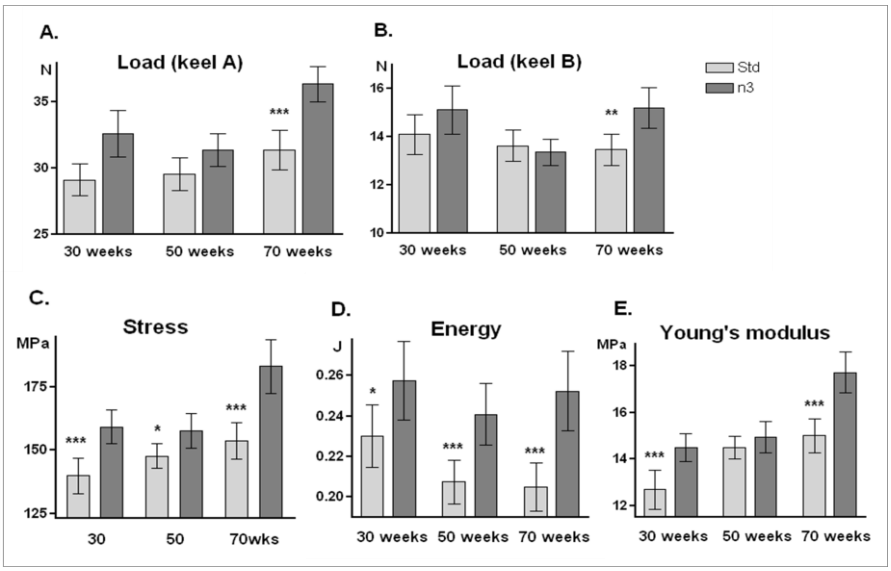

Fig. 4

Table 3. Statistical analysis of comparisons

	Overall Treatment effect (P)	**Bonferroni's Post-hoc test (P)**	**One way ANOVA (P)**
Breakage rates and severity			
By Dissection	0.001 n3<std	0.001 @50wks 0.011 @70wks	<0.0001
Severity of breaks	0.0012 n3<std	<0.05 @50wks	<0.0004
By Palpation	0.001 n3<std	0.028 @50wks	<0.0001
Keel Biomechanics			
Keel "A"	P=0.0002 n3>std	<0.001 @70wks	<0.004
Keel "B"	P<0.05	<0.01 @70wks	<0.0001
Tibia Biomechanics			
Ultimate Stress	0.002 n3>std	0.0001 @30wks 0.016 @50wks <0.0001 @70wks	<0.0001
Energy to break	0.001 n3>std	0.028 @30wks 0.0003 @50wks <0.0001 @70wks	<0.0001
Elastic Energy	0.003 n3>std	0.013 @70wks	0.03
Plastic Energy	0.002 n3>std	0.035 @30wks 0.0002 @50wks 0.0003 @70wks	<0.0001
Young's Modulus	0.012 n3>std	0.0004 @30wks <0.0001 @70wks	<0.0001
Histomorphometry (@70 weeks)			
TBV	Not done	0.0152	Not done

MBV	Not done	0.0018	Not done
TT	Not done	0.0096	Not done

Biochemistry

ALP	0.076 n3>std	NS	0.043
TRAP	NS	0.031 @30wks 0.038 @70 wks	0.0006
ProMMP-2	0.042 n3>std	0.049 @30wks	0.007
BMD	0.01 n3>std	0.002 @70wks	0.025
% mineral	0.01 n3>std	0.0002 @70wks	0.0003
OH-Pyr	<0.0001 n3<std	<0.0001 @30wks <0.0001 @50wks <0.0001 @70wks	<0.0001
L-Pyr	<0.0001 n3<std	<0.0001 @30wks 0.0002 @50wks <0.0001 @70wks	<0.0001
HLKNL	<0.0001 n3<std	0.0225 @30wks 0.0015 @70wks	0.0006
HHL	0.0008 n3<std	<0.001 @30wks <0.01 @70wks	<0.0001
HLNL	<0.0001 n3<std	NS	<0.0001
Intermediate: Mature X-links	<0.0001 n3>std	<0.0001 @30wks <0.0001 @50wks <0.0001 @70wks	<0.0001

3.6. Alkaline phosphatase (ALP)

Levels of ALP were higher in the humeri of the n3 fed hens at both 30 and 70wks, though this was not significant (Fig. 5A). There was a trend towards an overall treatment effect (p=0.076).

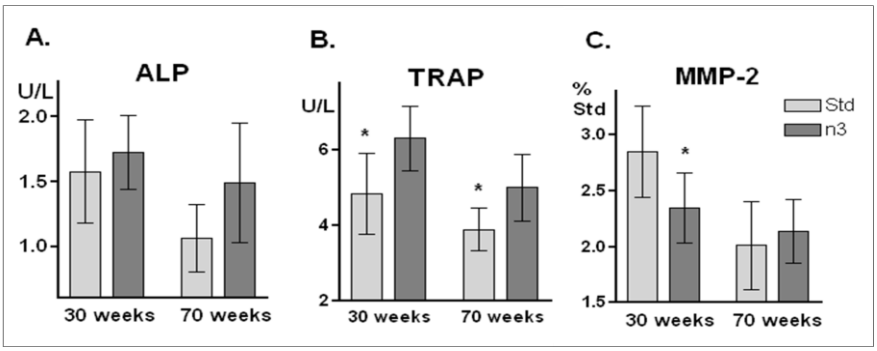

Fig. 5

3.7. Tartrate resistant acid phosphatase (TRAP)

Levels of TRAP in the humeri were significantly higher in the n3 flocks at 30 and 70wks ($p<0.05$), though the overall treatment effect was not found to be significant (Fig. 5B).

3.8. Pro-matrix metalloproteinase (proMMP)-2

There was a significant n3 treatment effect decreasing the expression of proMMP-2 in the humeri ($p<0.05$), with an individual time point decrease at 30 weeks ($p=0.049$). There was no apparent difference at 70 weeks (Fig. 5C).

3.9. Collagen cross-links

Intermediate (immature) cross-link: The intermediate cross link, hydroxylysino-5-ketonorleucine (HLKNL), was different in the n3 group ($p<0.0001$), being significantly lower at 30wks ($p<0.05$) and 70wks ($p<0.002$). Hydroxylysinonorleucine (HLNL) was different in the n3 treated group ($p<0.0001$), but no individual time point differences reached significance. Total intermediate crosslinks were lower in the n3 group ($p<0.0001$), significantly so at 30wks ($p<0.05$) and 70wks ($p<0.05$).

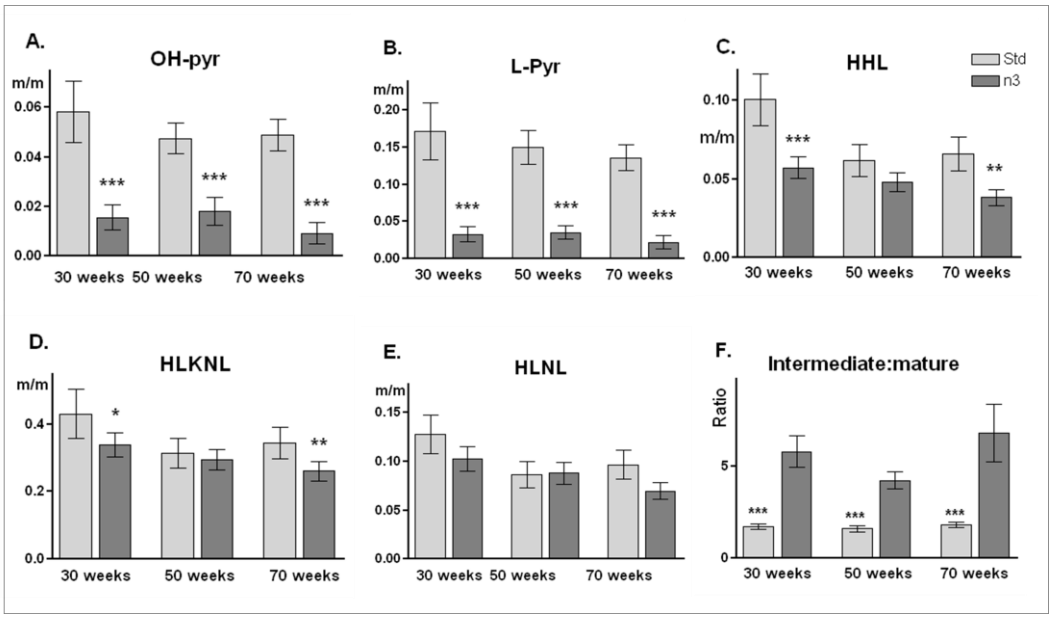

Fig. 6

Mature cross-links: Levels of the mature cross link HL-Pyr (Fig. 6A) were lower in the n3 fed birds ($p<0.0001$) and significantly so at 30, 50 and 70wks ($p<0.0001$). L-Pyr levels were also lower in the n3 group ($p<0.0001$), being significantly so at 30wk sand 70wks ($p<0.0001$), and at 50wks ($p-0.0002$). Histidinohydroxy-lysinonorleucine (HHL) levels were lower in the n3 group ($p<0.001$), significantly so at 30 and 70wks ($p<0.001$), and at 50wks ($p<0.01$). Total mature crosslinks excluding pyrrole were lower in the n3 group ($p<0.0001$), significantly so at 30wks ($p<0.0001$) 50wks ($p<0.001$) and 70wks ($p<0.0001$).

The ratio of intermediate to mature crosslinks was significantly greater with n3 treatment (Fig. 6F; $p<0.0001$), with all individual time point differences also significant ($p<0.0001$).

The levels of pyrrolic crosslink in both n3 and n6 groups (1.39m/m ±0.39) were markedly higher than previously reported in human[23] and avian[24] bone. There was no significant difference between the n3 and standard n6 diet groups.

4. Discussion

The principal outcome from this study is that dietary omega-3 polyunsaturated fatty acid (n3 PUFA), provided in the form of C18 alpha linolenic acid, markedly and significantly reduced the occurrence of keel bone fracture in laying hens. According to the dissection data, the levels of keel bone breakage in the n3 fed hens were 62% lower than the n6 group at 50wks and 42% lower at 70wks. Based on the proportion of the EU flock in free range systems in 2010, likely to be a

conservative estimate in light of the 2012 EU ban on battery cage systems, this would correspond to as many as 68 million fewer hens suffering from keel bone breakage in the EU each year. Current work within our group indicates that these fractures cause considerable pain, confirming the seriousness of this animal welfare issue and the benefits resulting from reducing fracture prevalence[25]. Fractures are also a serious economic problem, as broken keels are associated with reduced productivity through increased mortality[26], processors being unwilling to handle spent layers with bone fragments in the meat[27], decreased egg production and fragile shells[25]. The bone level data suggests that the benefits result from mechanical and structural changes within the bone. The bone strength (ultimate load and stress), toughness (energy to break) and rigidity (Young's modulus) all increase as a result of the n3 dietary supplementation, as do the bone volume, trabecular thickness and overall bone mineral density. The mechanism of action of n3 PUFAs relates to the mediators generated by PUFAs. These include prostaglandins, leukotrienes, thromboxanes, lipoxins, resolvins, maresins and protectins, and the mediators generated depend on which form of PUFA, n3 or n6, the carbon chain length, and other physiological parameters such as levels and state of cyclooxygenease (COX) and lipoxygenase (LOX). Omega-3 and n6 are essential fatty acids which must be obtained from the diet. The basic (C18) n3 is alpha linolenic acid (ALA), and the basic n6 is linoleic acid (LA). These are converted through a series of elongases and desaturases to the C20 eicosanoids - n3 eicosapenaenoic acid (EPA) and the n6 dihomo-gamma linolenic acid (DGLA) and arachidonic acid (AA). EPA is further converted to C22 docosahexaenoic acid (DHA). EPA (n3) is oxidised by COX and LOX to the series 3 prostaglandins and thromboxanes, and the series 5 leukotrienes. DGLA (n6) is oxidised to the series 1 prostaglandins, and AA (n6) to the series 2 prostaglandins and thromboxanes, and the series 4 leukotrienes. Furthermore, DHA and EPA are converted to resolvins, maresins and protectins, and AA to the lipoxins, all of which are important resolvers of inflammation (see supplementary Fig.1). All of these mediators have distinct effects on inflammation and bone metabolism. Overall the n3 mediators are somewhat less active and anti-inflammatory, and include more pro-resolving species, with the n6 mediators being more active and more inflammatory[10, 28]. Regulation of the levels of C20 and C22 mediator precursors is dependent on the activity of the desaturases and elongases, and upon the relative levels of ALA and LA. The levels of mediators are further regulated by the activities of COX and LOX. The conversion yield from C18 to C20/22 may be very low; our preliminary data suggests that this may be as low as 1% (data not shown). This implies that a diet with high levels of C20/22 PUFAs is likely to be considerably more biologically active than one with C18 alone, but it is possible that a diet rich in C20/22 would bypass the hen's natural and important regulatory mechanism in controlling mediator levels, and would therefore not necessarily be beneficial

A large number of animal and human studies have reported positive effects of n3 PUFAs in terms of increased bone formation, reduced bone resorption and protection from osteoporosis[29-31]. However, some have failed to demonstrate benefit, though this may be due to excessive levels of C20/22 PUFAs[32], or not supplementing with the optimal n3:n6 ratio[33], and claims from human studies are somewhat contradictory, possibly due to short time scales and confounding factors[34-36]. The mechanisms attributed for improvements in bone health relate to attenuation of mediators of osteoclastogenesis, such as PGE2, COX-2, IL1-β, TNFα and NF-κB, in particular via the E (EPA derived) and D (DHA derived) series of resolvins[9, 30, 37]. n3 PUFAs are also thought to increase osteoblast activity[10], although the precise mechanisms are not fully understood, and may be influenced by other factors such as age, concentration and bone site.

In this study we have demonstrated that a diet supplemented with n3 PUFA (ALA) significantly reduces keel bone breakage, improves the mechanical performance of the tibia, increases BMD and mineral content, and has a positive effect on the bone micro-architecture. The increased levels of ALP and TRAP, alongside the increase in the ratio of intermediate to mature crosslinks, suggest an overall increase in bone turnover. This contrasts with studies demonstrating benefits in treating osteoporosis by reducing bone remodelling [38, 39]. However, these studies were of established

osteoporosis, whereas the present study deals with improvements in bone strength and structure in preventing bone fragility and fractures, suggesting n3 may have a role in averting osteoporosis by increasing bone remodelling and net deposition, perhaps analogous to load and exercise. It may be that increased bone turnover prevents the accumulation of micro-cracks through an ongoing repair process[40], and that this is promoted in the n3 supplemented birds. This hypothesis is further supported by the apparent changes in the organic matrix and mechanical strength of the bone preceding those in the mineral component in the n3 birds. Reduced levels of proMMP-2 in the n3 treated group at 30wks suggest a mechanism that may account for the apparent net bone formation. However, MMP-2 is only one of the many proteases involved in bone turnover, and in any case this relationship is not apparent at 70wks.

As in humans, spontaneous osteoporosis in laying hens results from a decline in estrogen with age, but in sexually mature hens this is exacerbated by a switch in osteoblast deposition from structural bone to medullary bone which acts as a source of calcium for egg shell production[41, 42]. We have identified an increase in the thickness and total volume of trabecular bone in the n3 fed group, which, alongside higher levels of ALP, greater BMD and increased structural bone mineral content, indicate a change in the balance in remodelling in favour of increased structural bone formation, or perhaps a reduced rate of bone loss. Further work is needed to clarify these mechanisms.

In conclusion, this study has demonstrated the diets supplemented with n3 ALA can significantly reduce keel bone breakage rates in laying hens which equates to 50 million fewer keel bone breaks in the EU flock each year. Furthermore we have demonstrated n3 mediated modifications in the immediate upstream mechanisms of bone remodelling which indicate that dietary supplementation may provide benefits in preventing osteoporosis in other species, including post-menopausal osteoporosis in humans.

Acknowledgements

We would like to thank Noble Foods UK, and in particular Andrew Joret their Technical Director, for their assistance in this study.

Funding source

This work was funded by DEFRA and BBSRC. The funders had no role in the writing or submission of this article.

Figure Legends

Fig. 1. Ordinal scale of keel fracture severity, from 0 (no break) to 4 (severe break). Mechanical test sites for keel strength are shown for the manubrial spine (A) and the lateral surface (B).

Fig. 2. Keel bone fractures detected by dissection (A) and palpation (B) at age 30, 50 and 70 weeks. For dissection the overall treatment effect of n3 supplementation on reducing fracture rate prevalence was significant ($p<0.001$) with time point comparisons at 50 (** $p=0.01$) and 70 (*** $p=0.001$) weeks showing significant reductions. For palpation the overall treatment effect of n3 supplementation on reducing fracture rate prevalence was significant ($p=0.001$) with time point comparison at 50 weeks showing significant reductions (* $p<0.05$). Where keel fractures were present, severity also showed a significant reduction in the n3 group, with keel scores demonstrating a treatment effect ($p<0.002$), (C) showing severity distribution at 50 and 70 weeks (see fig.1 for definition of scores).

Fig. 3. Structural properties of the humeri improved with n3 supplementation. The n3 treated hens showed increased bone mineral density (BMD) (A) and mineral content (B) at 70 weeks (*** $p<0.0001$). Trabecular thickness (TT) (C) (*** $p=0.001$) and volume (D) (* $p<0.02$) and medullary bone volume (** $p<0.002$) also increased with n3 supplementation at 70 weeks.

Fig. 4. The strength of the keel bones at positions "A" and "B" (see fig.1) were increased with n3 supplementation (A and B), showing an overall treatment effect for keel "A" ($p=0.0002$) and keel "B" ($p<0.05$), and significant time point differences at 70 weeks (*** $p<0.01$ ** $p<0.001$). Mechanical properties of the tibia also improved with n3 supplementation. The n3 treated hens showed increased ultimate stress at breakage (C), with overall treatment effect ($p=0.002$), and significant time point comparisons at 30 (*** $p=0.0001$), 50 (* $p<0.02$) and 70 (*** $p<0.0001$) weeks. The n3 treated hens showed increased energy to break (D), with overall treatment effect ($p=0.001$), and significant time point comparisons at 30 (* $p<0.05$), 50 (*** $p<0.0005$) and 70 (*** $p<0.0001$) weeks. The n3 treated hens also showed increased Young's modulus (E), which is a measure of stiffness, with overall treatment effect ($p=0.02$), and significant time point comparisons at 30 (*** $p<0.0005$) and 70 (*** $p<0.0001$) weeks.

Fig. 5. Markers of bone turnover were modified with n3 supplementation. The n3 treated hens showed increased levels of alkaline phosphatase (ALP) (A), but the treatment effect failed to reach significance ($p=0.076$). Tartrate resistant acid phosphatase (TRAP) also increased with n3 supplementation (B) with no overall treatment effect, but significant time point comparisons at 30 weeks (* $p<0.05$) and 70 weeks (* $p<0.05$). The n3 treated hens showed lower levels of MMP-2 at 30 weeks (* $p<0.05$) and an overall treatment effect ($p<0.05$).

Fig. 6. Collagen crosslink profile was modified with n3 supplementation. The n3 treated hens showed a highly significant reduction in mature collagen crosslinks, hydroxylysyl pyridinoline (OH-pyr) ($p<0.0001$) (A), lysyl pyridinoline (L-pyr) ($p<0.0001$) (B) and histidinohydroxy-lysinonorleucine (HHL) ($p<0.001$) (C). Significant individual time point differences were seen for each pyridinoline crosslink at each time point ($p<0.0001$ for all except L-pyr at 50 weeks; $p=0.0002$). Individual time point differences for HHL did not reach significant. There were rather less marked n3 mediated reductions in the levels of the immature collagen crosslinks hydroxylysino-5-ketonorleucine (HLKNL) (D) and hydroxylysinonorleucine (HLNL) (E). Treatment effects for each were significant ($p<0.0001$), with individual time point comparisons significant for HLKNL at 30 ($p<0.05$) and 70 weeks ($p<0.002$), but no individual time point differences for HLNL. There was a relative increase in the overall proportion of immature crosslinks when compared with mature crosslinks (F) significant in all individual time point comparisons ($p<0.0001$) and an overall treatment effect ($p<0.0001$).

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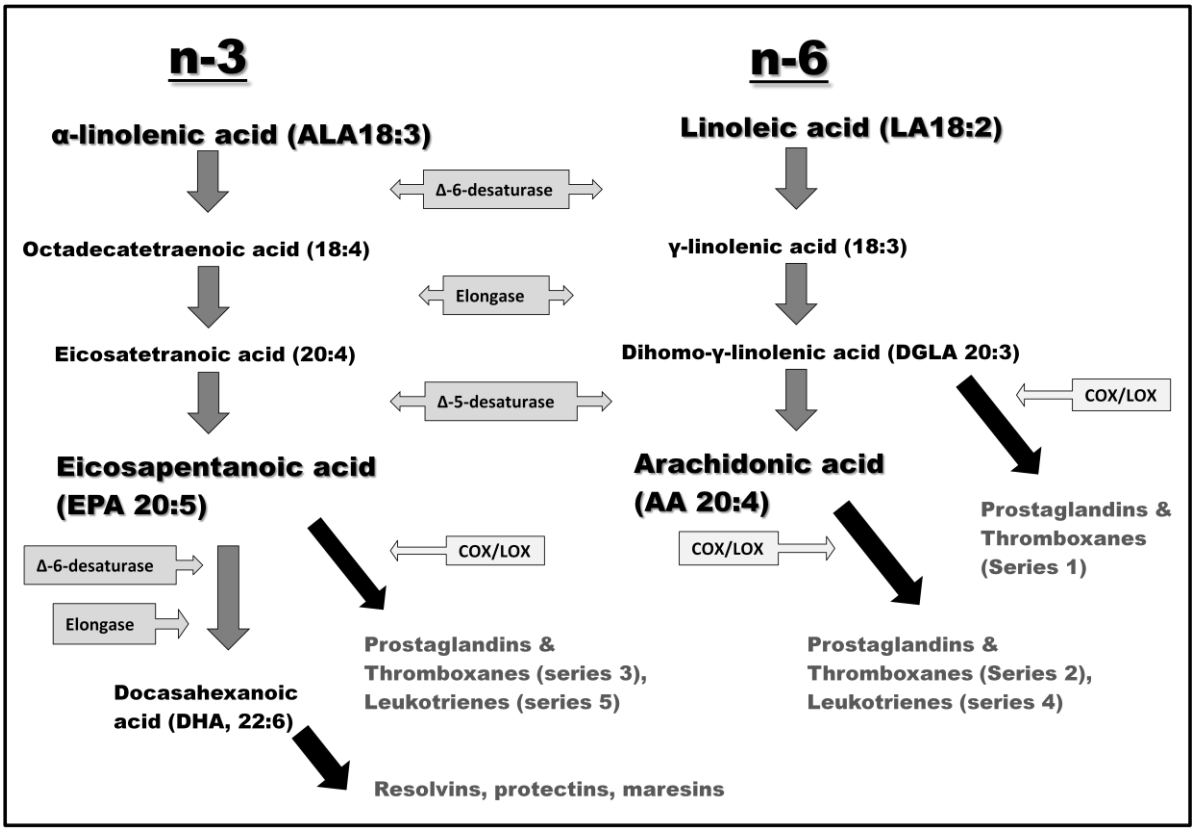
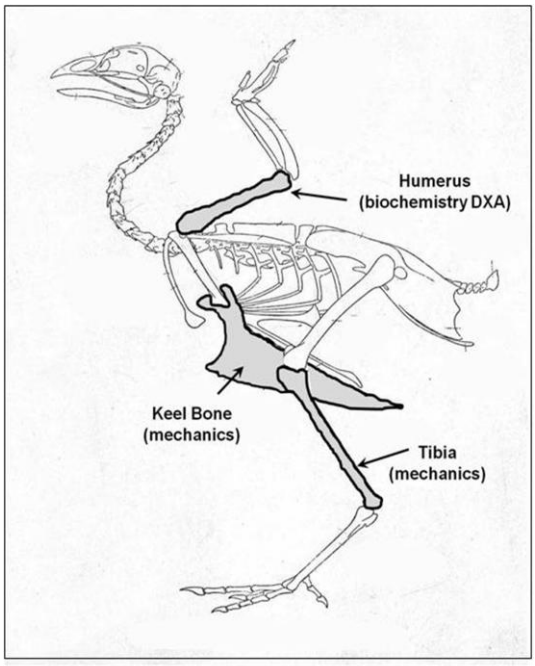
Supplementary figures



Supplementary Fig. S1



Supplementary Fig.S2

Table S1. Comprehensive data set. Mean ± SEM

	30 weeks		50 weeks		70 weeks	
	Std diet	n3 diet	Std diet	n3 diet	Std diet	n3 diet
Flock breakage Rates (%)						
By Dissection	5.18 ± 2.32 N=6	3.33 ± 2.11 N=6	59.40 ± 7.90 N=5	21.67 ± 3.07 N=6	65.00 ± 6.52 N=5	38.33 ± 5.43 N=6
By Palpation	5.83 ± 1.14 N=6	3.33 ± 0.96 N=6	48.40 ± 8.13 N=5	20.00 ± 2.13 N=6	44.40 ± 5.95 N=5	33.17 ± 5.11 N=6
Severity of Keel Breaks (grade)	1.3 ± 0.58 N=3	1.5 ± 0.71 N=2	2.1 ± 0.88 N=59	1.4 ± 0.64 N=28	1.8 ± 0.90 N=65	1.4 ± 0.72 N=46
Keel Biomechanics						
Keel "A" (N)	29.1 ± 4.6 N=59	32.6 ± 6.7 N=59	29.5 ± 6.2 N=99	31.4 ± 6.8 N=120	31.4 ± 7.4 N=99	36.4 ± 7.3 N=120
Keel "B" (N)	14.1 ± 3.2 N=59	15.1 ± 3.8 N=59	13.6 ± 3.3 N=99	13.4 ± 3.0 N=119	13.5 ± 3.3 N=98	15.2 ± 4.6 N=120
Tibia Biomechanics						
U Stress (MPa)	139.9 ± 3.5 N=48	159.1 ± 3.3 N=61	147.5 ± 2.5 N=114	157.6 ± 3.4 N=66	153.6 ± 3.6 N=77	182.9 ± 5.34 N=61
U Energy (J)	0.2300 ± 0.0075 N=30	0.2573 ± 0.0096 N=53	0.2074 ± 0.0054 N=112	0.2407 ± 0.0076 N=66	0.2049 ± 0.0060 N=79	0.2521 ± 0.0098 N=64
E Energy (J)	0.01739 ± 0.0008 N=57	0.01890 ± 0.0008 N=61	0.01950 ± 0.0006 N=114	0.0189 ± 0.0007 N=66	0.01810 ± 0.0007 N=76	0.02107 ± 0.0009 N=60
P Energy (J)	0.2130 ± 0.0077 N=30	0.2414 ± 0.0087 N=58	0.1878 ± 0.0052 N=112	0.2218 ± 0.0075 N=66	0.1874 ± 0.0060 N=79	0.2268 ± 0.0094 N=61
Young's Mod (MPa)	12.68 ± 0.41 N=48	14.51 ± 0.30 N=61	14.50 ± 0.25 N=113	14.95 ± 0.33 N=65	15.00 ± 0.36 N=77	17.72 ± 0.44 N=60
Histomorphometry						
TBV (%)	ND	ND	ND	ND	20.59 ± 0.9778 N=39	24.76 ± 1.328 N=20
MBV (%)	ND	ND	ND	ND	15.25 ± 1.424 N=39	20.54 ± 0.7577 N=16
TT (µm)	ND	ND	ND	ND	46.89 ± 2.017 N=40	57.44 ± 3.855 N=20
Biochemistry						
ALP (U/L)	1.578 ± 0.197 N=34	1.727 ± 0.142 N=43	ND	ND	1.061 ± 0.130 N=44	1.488 ± 0.229 N=42
TRAP (U/L)	4.843 ±	6.315 ±	ND	ND	3.885 ±	5.004 ±

	0.528 N=33	0.423 N=42			0.281 N=44	0.443 N=43
ProMMP-2	2.848 ± 0.201 N=33	2.344 ± 0.156 N=39	ND	ND	2.009 ± 0.195 N=42	2.136 ± 0.139 N=45
BMD	203.8 ± 6.4 N=36	199.5 ± 3.7 N=43	ND	ND	190.0 ± 2.5 N=44	205.4 ± 3.1 N=45
% mineral	66.17 ± 0.88 N=34	67.00 ± 1.62 N=41	ND	ND	62.07 ± 2.03 N=42	72.11 ± 1.58 N=44
OH-Pyr	0.05816 ± 0.00607 N=38	0.01552 ± 0.00246 N=50	0.04737 ± 0.00307 N=54	0.01813 ± 0.00277 N=40	0.04862 ± 0.00319 N=47	0.00905 ± 0.00215 N=40
L-Pyr	0.1718 ± 0.0188 N=38	0.03264 ± 0.0052 N=50	0.1496 ± 0.0113 N=54	0.0349 ± 0.0045 N=40	0.1360 ± 0.0085 N=47	0.02203 ± 0.0043 N=40
HLKNL	0.4303 ± 0.0357 N=38	0.3386 ± 0.0173 N=50	0.3126 ± 0.0221 N=54	0.2945 ± 0.0148 N=40	0.3440 ± 0.0228 N=47	0.2602 ± 0.0143 N=39
HHL	0.1005 ± 0.0082 N=38	0.05716 ± 0.0034 N=50	0.06185 ± 0.0052 N=54	0.0480 ± 0.0029 N=40	0.06589 ± 0.0053 N=47	0.03818 ± 0.0024 N=39
HLNL	0.1279 ± 0.0098 N=38	0.1027 ± 0.0061 N=48	0.0867 ± 0.0067 N=54	0.0879 ± 0.0054 N=40	0.0966 ± 0.0072 N=47	0.0695 ± 0.0042 N=39
Tot X-links	0.8888 ± 0.0707 N=38	0.4428 ± 0.0246 N=49	0.6583 ± 0.0427 N=54	0.4833 ± 0.0242 N=40	0.6910 ± 0.0399 N=47	0.3993 ± 0.0231 N=39
Intermediate: Mature X-links	1.708 ± 0.074 N=38	5.802 ± 0.429 N=50	1.594 ± 0.075 N=54	4.238 ± 0.236 N=40	1.804 ± 0.070 N=47	6.821 ± 0.770 N=40